Pressure-dependent Activity of Dihydrofolate Reductase from a Deep-sea Bacterium *Shewanella violacea* Strain DSS12

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A new dihydrofolate reductase (svDHFR) was purified from a deep-sea bacterium, *Shewanella violacea* strain DSS12, isolated from the Ryukyu Trench (5110 m). In contrast with *E. coli* DHFR, the enzyme activity of svDHFR increased with increasing hydrostatic pressure up to 100 MPa, suggesting that the enzyme kinetics and structural fluctuation of svDHFR are adapted to a high-pressure environment.

What underlies the adaptation of microorganisms to the deep sea, an extreme environment with a high hydrostatic pressure? Since this is a basic problem for understanding their evolution process, the genes of a large variety of deep-sea bacteria have been identified.[1–3] However, the mechanism of bacteria adaptation for the deep sea is not well understood at the protein level,[4,5] probably because it is difficult to construct overexpression and purification systems of proteins from such microorganisms.

Dihydrofolate reductase (DHFR) is an excellent target enzyme to investigate the pressure-adaptation mechanism of proteins because this enzyme is essential in living cells. DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), which is an important precursor of a cofactor for the synthesis of purine nucleoside. It is known that DHFR from *Escherichia coli* (ecDHFR) is highly flexible and that its structure is sensitively influenced by pressure.[6,7] This study aimed to characterize a new DHFR (svDHFR) from a deep-sea bacterium, *Shewanella violacea* strain DSS12, which was isolated from the Ryukyu Trench (at a depth of 5110 m).[8,9] This bacterium can grow under hydrostatic pressures from 0.1 to 70 MPa with optimal growth occurring at 30 MPa.[10] We have cloned its gene, and constructed overexpression and purification systems of the svDHFR using *E. coli* cells. Its structure and function were compared with those of ecDHFR under pressures up to 250 MPa.

Genomic DNA of *S. violacea* strain DSS12 was kindly provided by Dr. Nogi of JAMSTEC, and was digested by *Eco*RI for a PCR template. The PCR reaction was carried out with two primers, 5′AGGAACTTCCATGAAAATCGCCATCATAGC3′ (forward) and 5′GAGGATCCTTAACATTTTTTAACCAA GT3′ (reverse), and KOD-plus DNA polymerase (TOYOBO). The PCR product was purified with Wizard SV gel and PCR clean-up system (Promega), ligated to pUC118 vector digested by *Sma*I, and then transformed to *E. coli* HB101 competent cells (TAKARA). The transformants were selected by an LB-plate containing 100 μg/mL ampicilin and 5 μg/mL trimethoprim and checked by DNA sequencing.

Sv-DHFR protein was purified from *E. coli* BL21 strain containing the above expression plasmid using a methotrexate-agarose (Sigma) affinity column and a DE-52 (Whatman) anion-exchanger column. The protein concentration was determined using a molar extinction coefficient of 18100 $M^{-1}cm^{-1}$ at 280 nm, which was calculated from the fluorescence intensity in a solution containing 6 M urea.[11]

Far-ultraviolet circular dichroism (CD) spectra of DHFRs were measured at 15 °C using a Jasco J-720W spectropolarimeter. The solvent conditions were 20 mM Tris-hydrochloride (pH 7.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol. The protein concentration was 20 μM.

The enzyme reaction under atmospheric and high pressures was quantified with a Shimadzu UV-1600PC spectrophotometer equipped with a high-pressure absorbance cell unit (Teramecs PCR-400) and a hand pump (Teramecs TP-500). The temperature was maintained at 25 ± 0.1 °C using a circulating thermobath (NESLAB RTE-111). The buffer was the same as that used in CD measurements and the enzyme was present at concentrations of 1–2 nM. The concentrations of NADPH and DHF were 50 and 40 μM, respectively, which were much higher than their K_m values (1–2 μM).[6] The rate of the enzyme reaction under atmospheric pressure was calculated using a differential molar extinction coefficient of 11800 $M^{-1}cm^{-1}$ at 340 nm.[12] Under high pressures this value was corrected for the pressure-dependent variations in the absorptions of the solutions of substrates (NADPH and DHF) and products ($NADP^+$ and THF).

Figure 1 shows SDS-PAGE of the purified svDHFR protein. Although some light bands appear at higher molecular weights, a highly purified band of svDHFR is observed at 17.6 kDa, which corresponds to 160 amino acid residues.

Figure 2 shows far-ultraviolet CD spectra of svDHFR and ecDHFR at 15 °C and pH 7.0. The CD spectrum of svDHFR is

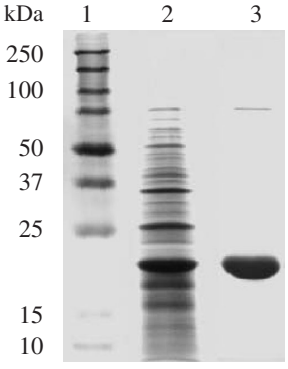

Figure 1. SDS-PAGE of the svDHFR. Lane 1: Marker. Lane 2: Whole proteins of *E. coli* BL21 strain containing the svDHFR expression plasmid. Lane 3: Purified svDHFR protein.

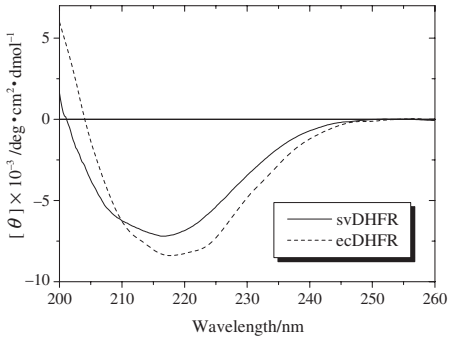

Figure 2. Far-ultraviolet circular dichroism spectra of svDHFR and ecDHFR at 15 °C and pH 7.0.

slightly blue-shifted and decreased in intensity compared with ecDHFR. This result suggests that the secondary structure of svDHFR is similar to that of ecDHFR, as expected from a 55% homology in the primary structure between the two DHFRs.

Figure 3 shows the relative activities of the DHFRs as a function of hydrostatic pressure. The enzyme activity of svDHFR increases with pressure, to at most 30% at 100 MPa, and then gradually decreases although maintains a higher activity at 250 MPa than at atmospheric pressure. This is significantly different from the behavior of ecDHFR, which exhibits a monotonous decrease in the activity even under low pressure. Therefore, it can be expected that svDHFR has distinguished characteristics in enzyme kinetics and structural fluctuation to adapt itself to deep-sea conditions.

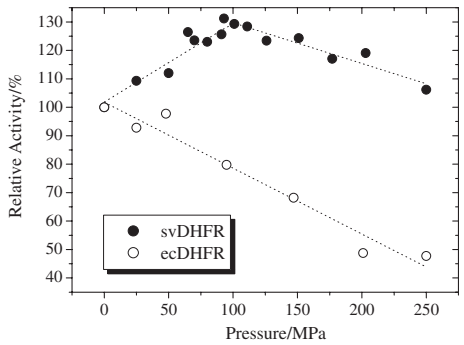

Figure 3. Pressure dependence of the enzyme activities of svDHFR and ecDHFR. Dashed lines represent only the trends of the data.

The activation volume of enzyme reaction, $\Delta V^{\ddagger}$, was calculated from the following equation since the rate constant, k, can be regarded as the initial velocity, v, of the enzyme reaction at the saturated concentration of substrate:

$$\Delta V^{\ddagger} = \partial(-RT \ln k)/\partial P = \partial(-RT \ln v)/\partial P$$

where R is the gas constant, T the temperature, and P the pressure. The obtained $\Delta V^{\ddagger}$ values are listed in Table 1. The negative $\Delta V^{\ddagger}$ value of svDHFR under pressures below 100 MPa, as expected from the positive slope in Figure 3, means that the activated state has a smaller volume than the reactant in the catalytic reaction coordinate. At pressures above 100 MPa this is reversed, as shown by the positive $\Delta V^{\ddagger}$ value (also for ecDHFR). These results predict that svDHFR, being highly flexible at pressures below 100 MPa, changes its conformation to be more rigid (like ecDHFR) at higher pressures although the protein may be denatured at pressures above a few hundred MPa. High-pressure NMR of ecDHFR revealed the presence of two conformers whose populations were sensitively influenced by pressure.[7] EcDHFR catalyzes the NADPH-linked reduction of DHF to THF through a cycle of five intermediate states: DHFR–NADPH, DHFR–NADPH–DHF, DHFR–$NADP^+$–THF, DHFR–THF, and DHFR–NADPH–THF; involving some equilibrium states such as DHFR–$NADP^+$.[13] The ternary complex DHFR–NADPH–DHF exists only transiently because the hydride transfer from NADPH to DHF is very rapid. Determining the volume changes in each process and the rate-limiting process, which is in progress, should provide more detailed understanding of the adaptation mechanism of the enzyme reaction.

Table 1. The activation volume ($\Delta V^{\ddagger}$) of the enzyme reaction of svDHFR and ecDHFR at 25 °C and pH 7.0

Protein	Pressure/MPa	$\Delta V^{\ddagger}$/mL/mol
svDHFR	0.1–100	-5.8 ± 0.8
	100–250	2.7 ± 0.4
ecDHFR	0.1–250	8.1 ± 0.8

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